

Bioinspired Vesicle Restraint and Mobilization Using a Biopolymer Scaffold

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Biology employs vesicles to package molecules (e.g., neurotransmitters) for their targeted delivery in response to specific spatiotemporal stimuli. Biology is also capable of employing localized stimuli to exert an additional control on vesicle trafficking; intact vesicles can be restrained (or mobilized) by association with (or release from) a cytoskeletal scaffold. We mimic these capabilities by tethering vesicles to a biopolymer scaffold that can undergo (i) stimuli-responsive network formation (for vesicle restraint) and (ii) enzyme-catalyzed network cleavage (for vesicle mobilization). Specifically, we use the aminopolysaccharide chitosan as our scaffold and graft a small number of hydrophobic moieties onto its backbone. These grafted hydrophobes can insert into the bilayer to tether vesicles to the scaffold. Under acidic conditions, the vesicles are not restrained by the hydrophobically modified chitosan (hm-chitosan) because this scaffold is soluble. Increasing the pH to neutral or basic conditions allows chitosan to form interpolymer associations that yield a strong, insoluble restraining network. Enzymatic hydrolysis of this scaffold by chitosanase cleaves the network and mobilizes intact vesicles. Potentially, this approach will provide a controllable means to store and liberate vesicle-based reagents/therapeutics for microfluidic/medical applications.

Introduction

Biology routinely uses vesicles for intercellular communication. For instance, Figure 1a illustrates that signaling between pre- and postsynaptic cells in the nervous system is mediated by the release of neurotransmitter molecules from vesicles located in the active zone of the presynaptic cell. Fusion between the vesicle and cytoplasmic membranes during exocytosis results in the release of neurotransmitter from the presynaptic cell into the synaptic cleft, whereas the postsynaptic cell recognizes this signal by the selective binding of the transmitter to its receptor. In addition to the free vesicles located in the active zone, the presynaptic cell also possesses a reserve pool of transmitter-containing vesicles that are tethered to (i.e., “restrained”) by the cytoskeleton. Figure 1b shows that these restrained vesicles are tethered to the filamentous actin scaffold by the protein synapsin.^{1–3} These restrained vesicles are mobilized (i.e., “recruited”) in response to cellular needs by the enzyme-catalyzed phosphorylation of synapsin.^{4,5} Thus, dephosphorylation and phosphorylation of synapsin provide the localized cues to restrain or mobilize these reserve vesicles.

Figure 1c shows our approach to mimic in vitro the processes of vesicle restraint and mobilization. As evident from this schematic, our approach mimics the capabilities although not the mechanistic details of biological vesicle restraint and mobilization. We use the aminopolysaccharide chitosan as our

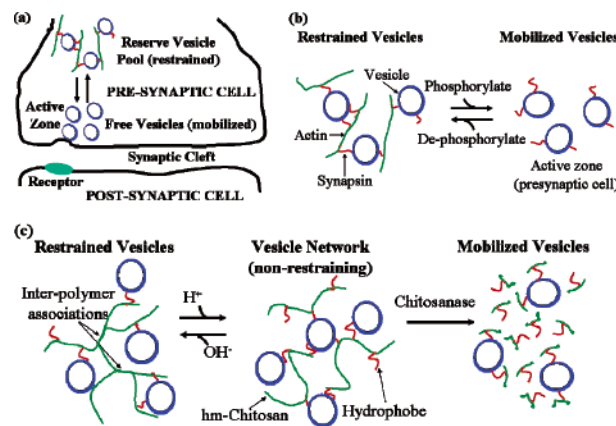


Figure 1. Schematic of biological and bioinspired processes to restrain and mobilize vesicles. (a) Intercellular signaling in the nervous system relies on the release of neurotransmitter molecules from vesicles in presynaptic nerve cells. (b) Protein phosphorylation and dephosphorylation provide the localized cues to mobilize and restrain vesicles in presynaptic cells. (c) Bioinspired approach to mobilize and restrain vesicles.

scaffold in place of the proteinaceous actin scaffold. Tethering of vesicles to our scaffold is achieved using *n*-dodecyl hydrophobes grafted onto the chitosan backbone. These grafted hydrophobes can insert into the vesicle’s bilayer to mediate vesicle–chitosan tethering in place of synapsin-mediated vesicle–actin tethering. As suggested by the central illustration in Figure 1c, each hydrophobically modified chitosan (hm-chitosan) chain has multiple hydrophobes on its backbone and can interconnect multiple vesicles into a 3D vesicle network.⁶ This initial vesicle network, which is formed under acidic conditions, offers some of the features of a reserve vesicle pool as the vesicles are tethered to a scaffold. As will be discussed, however, this acidic vesicle network is not sufficiently robust to restrain the vesicles.

To restrain the vesicles, the left illustration in Figure 1c shows that we propose to enlist the pH-responsive network-forming

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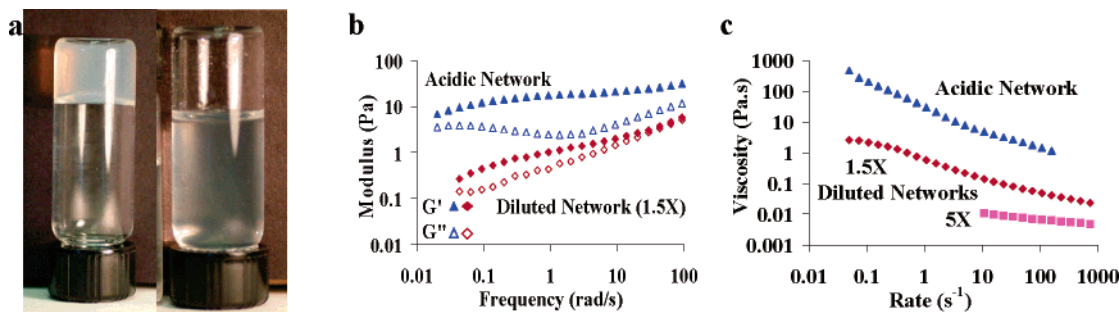


Figure 2. Acidic vesicle–hm-chitosan gel network can be diluted. (a) Photographs show that the acidic vesicle–hm-chitosan network is a gel that can support its weight upon vial inversion (left photograph), whereas dilution results in a solution (right photograph). (b) Dynamic rheological measurements show that dilution weakens the acidic vesicle gel network. (c) Steady-shear measurements show that dilution converts the acidic vesicle gel network into a solution.

properties of chitosan. Chitosan is a linear copolymer composed of glucosamine (typically over 80%) and *N*-acetylglucosamine residues. At low pH, the primary amines of the glucosamine residues are protonated, conferring a positive charge to chitosan. Under these acidic conditions (pH less than about 6), chitosan is a water-soluble polyelectrolyte. With increasing pH, the primary amines become progressively less charged, interpolymer repulsions are reduced, and chitosan can form interpolymer associations that lead to the formation of a robust, 3D hydrogel network. Importantly, chitosan's transition between soluble chains and an insoluble network occurs at pH values between 6 and 6.5.^{7–12} Thus, we propose that a pH increase to near-neutral or basic conditions can serve as the cue to restrain our vesicles. The right illustration in Figure 1c shows that we propose to mobilize our vesicles by the enzymatic hydrolysis of our scaffold using chitosanase.

The goal of this study is to demonstrate that the pH and chitosanase cues proposed in Figure 1c do in fact lead to the restraint and mobilization of our vesicles.

Materials and Methods

Chitosan from crab shells (reported by the supplier to be 85% deacetylation with a molecular weight of 370 000), *n*-dodecyl aldehyde, cetyl trimethylammonium tosylate (CTAT), sodium dodecyl benzene sulfonate (SDBS), and chitosanase (specific activity of 205 U/mg) were all purchased from Sigma-Aldrich Chemicals.

Hydrophobically modified chitosan (hm-chitosan) was prepared by reacting *n*-dodecyl aldehyde with chitosan using Schiff base chemistries^{13–19} as reported previously.⁶ Specifically, we reacted *n*-dodecyl aldehyde with chitosan (0.025 molar ratio of aldehyde to glucosamine residue) in a water–ethanol mixture and added sodium cyanoborohydride to reduce the Schiff base. The hm-chitosan was precipitated by raising the pH, and the precipitate was sequentially washed with ethanol and then deionized water four times. The resulting hm-chitosan was redissolved in 1% acetic acid.

Surfactant vesicles were prepared by mixing CTAT and SDBS (70:30 on a mass basis) with deionized water and gently stirring for 2 days. The presence of the vesicles in the mixture was detected by dynamic light scattering (DLS) measurements. To prepare the acidic vesicle–hm-chitosan network, the vesicle solution and hm-chitosan solution were mixed to the desired composition and held at 50 °C for 2 h. This acidic vesicle–hm-chitosan mixture was observed to form a gel network upon cooling, and this network was stored at 4 °C.

Rheological properties were measured using a Rheometric RDA III strain-controlled rheometer. All experiments were performed at 25 °C using a cone-and-plate with 25-mm diameter and 0.02-rad cone angle. Dynamic measurements were performed at a constant strain of 5%, which was determined by dynamic strain sweep experiments. When dynamic measurements were used to monitor the chitosanase-catalyzed cleavage of the acidic vesicle–hm-chitosan network, the enzyme (0.6 U/mL of gel) was briefly mixed with the

gel network, and the gel was then loaded onto the rheometer. Dynamic measurements of this degrading network were performed until values of G' and G'' became too small to be accurately measured.

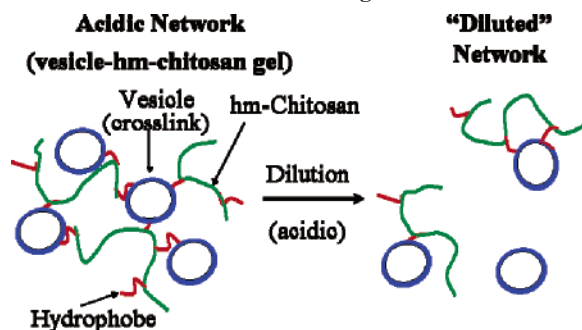
Dynamic light scattering (DLS) was used to determine vesicle size. All measurements were performed using a Photocor-FC light-scattering instrument with a 5-mW laser light source at 633 nm with a scattering angle of 90°. A logarithmic correlator was used to measure the intensity autocorrelation function. Hydrodynamic radius distributions were extracted from the correlation functions using the Dyna-LS software package supplied by Photocor. The mean of the distribution matched well with the value obtained by conventional data analysis using the method of cumulants coupled with the Stokes–Einstein equation.²⁰

Results and Discussion

Nonrestraining Vesicle Network (Acidic Conditions). The acidic vesicle–hm-chitosan network was formed by combining the hm-chitosan and vesicle solutions to achieve final concentrations of 0.55 wt % polymer and 0.5 wt % surfactant. The left photograph in Figure 2a shows that this acidic vesicle–hm-chitosan network behaves as a gel because it can support its weight upon vial inversion. On the basis of previous small-angle neutron scattering (SANS) studies of this gel,⁶ we attribute gel formation to the noncovalent cross linking of hm-chitosan chains by the vesicles, as shown in the central illustration of Figure 1c. (We should note that an equivalent mixture of vesicles and unmodified chitosan does not lead to the formation of such a gel network under acidic conditions.⁶) This acidic vesicle–hm-chitosan gel network is only weakly effective at restraining vesicles. When water is added to the sample such that the final pH remains below 4, the vesicle–hm-chitosan network becomes

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Scheme 1. Disruption of Acidic Vesicle–hm-Chitosan Gel Network by Dilution Due to the Loss of Vesicle-Mediated Cross Linking



progressively diluted. Initially, dilution weakens the gel network, whereas at higher dilution the network is transformed into a viscous solution that can no longer support its own weight, as shown by the right photograph in Figure 2a.

The visual observation that dilution can break the acidic vesicle gel network is supported by rheological measurements. Dynamic measurements shown in Figure 2b demonstrate that the acidic vesicle–hm-chitosan network is a weak gel. Specifically, the elastic modulus (G') is rather small (~ 10 Pa) but somewhat larger than the viscous modulus (G''), and both moduli show a weak dependence on frequency. When the acidic vesicle–hm-chitosan gel network is diluted with a small amount of water (2 parts gel to 1 part water), Figure 2b shows that the sample becomes more liquidlike— G' and G'' become similar, and both moduli become frequency-dependent. When the acidic vesicle–hm-chitosan network is diluted with larger amounts of water, the sample becomes a liquid, and its properties can no longer be reliably measured by dynamic rheology. Steady-shear measurements in Figure 2c further support the results from dynamic testing. Specifically, dilution of the acidic vesicle–hm-chitosan gel network results in substantial decreases in viscosity and a reduced dependence on shear rate.

Scheme 1 shows our interpretation of the results in Figure 2. At the left in Scheme 1 is a percolating network formed by interactions between hm-chitosan and vesicles. Several studies have indicated that hydrophobically modified water-soluble polymers can interact with vesicles by insertion of the hydrophobes into the bilayer.^{21–30} At sufficiently high concentrations, the polymer can bridge vesicles, and the vesicles can serve as network junctions to yield an elastic gel.^{21–23,26–29} In our earlier studies, we provided rheological, microstructural (i.e., cryo-TEM), and nanostructural (i.e., SANS) evidence to support the acidic vesicle–hm-chitosan network structure shown at the left in

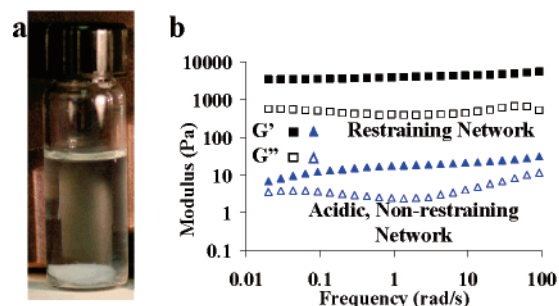


Figure 3. Base treatment converts the weak vesicle–hm-chitosan network into a restraining network. (a) Photograph showing that base treatment converts the initial network into two phases—a clear solution and an insoluble, opaque gel at the bottom of the vial. (b) Dynamic rheological measurements show the base-treated restraining gel network is considerably stronger than the acidic, nonrestraining gel network.

Scheme 1.^{6,31} We believe that dilution of the acidic vesicle–hm-chitosan gel reduces the density of vesicle network junctions such that there are insufficient cross links to form a volume-filling 3D network. Thus, because the chitosan scaffold is soluble under acidic conditions and the grafted hydrophobes can rapidly exchange with the bilayer, this acidic network is unable to restrain the vesicles.

pH Increase to Generate a Restraining Network. To create a true restraining network, the left illustration in Figure 1c shows that we propose a pH increase to induce the formation of interpolymer associations that can serve as more robust physical network junctions. Experimentally, we added a basic NaOH solution to the acidic vesicle–hm-chitosan network to ensure that the final pH exceeded 8. The photograph in Figure 3a shows that this base addition causes the sample to separate into two phases, an opaque gel phase visible at the bottom of the vial and a transparent and nonviscous supernatant solution. Vesicles were not detected in the supernatant using dynamic light scattering (DLS), suggesting that the vesicles are retained in the opaque gel. This suggestion is further supported by DLS analysis that shows that intact vesicles can be recovered from the insoluble, opaque gel (see below). These observations indicate that chitosan's interpolymer associations yield a sufficiently robust and insoluble network to restrain the tethered vesicles.

We recovered the insoluble, opaque gel obtained by base treatment and performed rheological measurements to compare the mechanical properties of this restraining gel network with those of the acidic vesicle–hm-chitosan gel network. The dynamic rheological measurements in Figure 3b demonstrate that this restraining network is much stronger than the acidic nonrestraining network. Specifically, G' for this restraining network is approximately 100-fold larger than that of the acidic network. Steady-shear rheological measurements (not shown) further support the results from dynamic rheology by showing that the restraining network has a yield stress and a larger viscosity than the acidic, nonrestraining vesicle–hm-chitosan network.

Enzymatic Scaffold Cleavage for Vesicle Mobilization. The right illustration in Figure 1c proposes that the chitosan-cleaving enzyme chitosanase can hydrolyze the scaffold to mobilize the vesicles. Chitosanase treatment is visually observed to convert the weak, acidic vesicle–hm-chitosan gel network into a low-viscosity solution within a few hours. The rheological measurements in Figure 4a confirm this observation and show that the enzyme-catalyzed gel breakage occurs over the course of an

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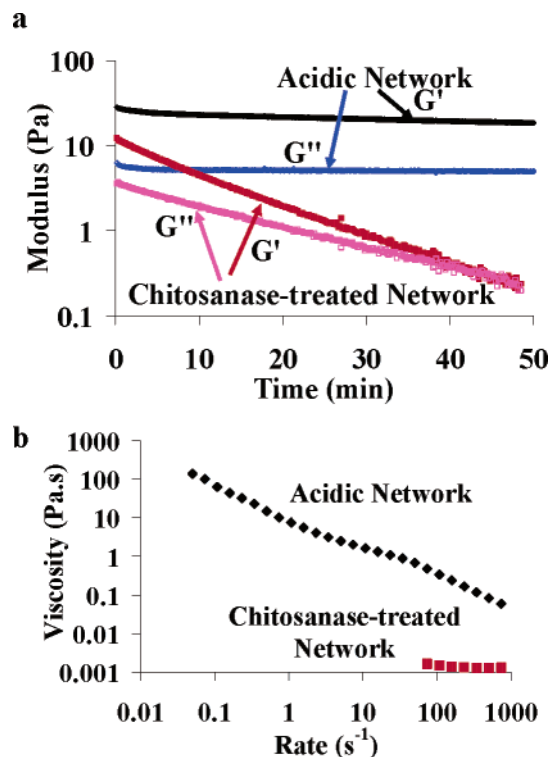


Figure 4. Chitosanase-catalyzed cleavage of the acidic vesicle-hm-chitosan gel network. (a) Time course of network cleavage using dynamic rheology. (b) Steady-shear rheological data comparing the initial acidic vesicle network and the chitosanase-treated network.

hour. Figure 4b shows that after chitosanase treatment the sample shows liquidlike rheology with Newtonian behavior and low viscosity. This behavior can be contrasted with that of the initial acidic vesicle network that has high viscosity and is shear-thinning. We should note that in addition to breaking the weak, acidic vesicle network, chitosanase can break the base-treated restraining network although enzymatic cleavage of this strong gel is slower and sensitive to buffering (i.e., chitosanase is inactivated under basic conditions).

Demonstration That Mobilized Vesicles Are Intact. To show that intact vesicles are mobilized upon chitosanase-catalyzed network cleavage, we performed dynamic light scattering (DLS) measurements. Specifically, we compared the results for our original vesicle solution (before adding hm-chitosan) with results for an acidic vesicle-hm-chitosan network after treatment with chitosanase. The similarity in the intensity autocorrelation functions in Figure 5a suggests that the original and mobilized vesicles are similar. The analysis of the particle size distribution for the original vesicles is shown in Figure 5b and indicates a polydisperse population of vesicles with a mean radius of 87 nm. Figure 5c shows that the chitosanase-mobilized vesicles have a somewhat narrower size distribution and a mean radius of 77 nm. These results confirm that intact vesicles are mobilized by chitosanase treatment of the acidic vesicle-hm-chitosan network. We should note an additional experiment we performed to indicate that the vesicles remain intact in the “insoluble” restraining network (data not shown). In this experiment, an insoluble restraining network was acidified and then treated with chitosanase to mobilize the vesicles. DLS measurements of these solutions showed a mean radius of 84 nm. Thus, intact vesicles can be mobilized from both the acidic and the restraining networks.

The effect of hm-chitosan on vesicle morphology is interesting. Others have shown that interactions between vesicles and hydrophobically modified polymers can result in reductions in

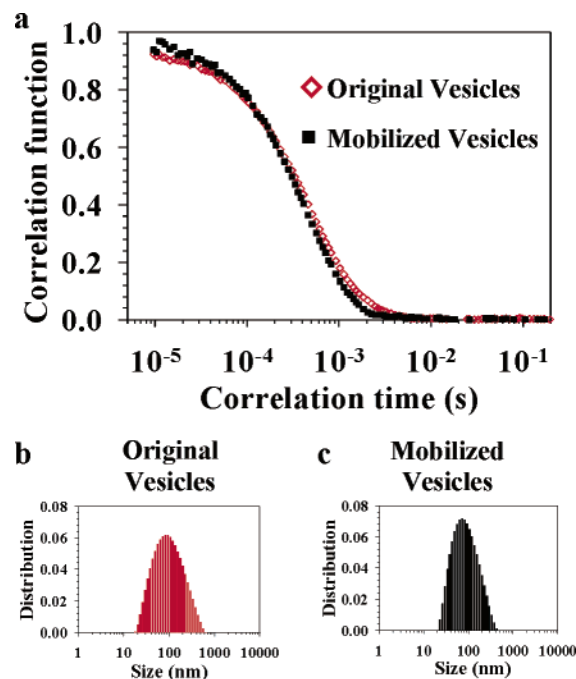


Figure 5. Evidence that intact vesicles are mobilized by chitosanase-catalyzed cleavage. (a) Dynamic light scattering results showing the intensity autocorrelation function ($g_2(\tau) - 1$) vs correlation time (τ) for the original vesicle solution and vesicles liberated from the acidic vesicle-hm-chitosan network by chitosanase. Corresponding particle size distributions for (b) the original vesicles and (c) the chitosanase-mobilized vesicles.

vesicle size²⁵ and changes in vesicle shape.^{27,28,30} In previous studies with SANS and cryo-TEM, we observed that the addition of hm-chitosan to the vesicles results in a substantial reduction in vesicle diameter (120 to 40 nm) and a transition from unilamellar to bilamellar vesicles.^{6,31} Figure 5 shows a less-dramatic change in vesicle size between our initial vesicles (before contact with polymer) and vesicles after they have been released by chitosanase treatment. Because others have observed vesicle size to depend on polymer molecular weight,²⁵ it would be interesting to know whether the vesicle size increases upon hm-chitosan cleavage. We hope to have the opportunity to perform SANS measurements to answer this question.

Conclusions

We report an approach to vesicle restraint and mobilization that is analogous to biological mechanisms in the following ways: (i) we use a biopolymer scaffold; (ii) we tether the vesicles to the scaffold; and (iii) we trigger vesicle restraint and mobilization by chemical cues. Specifically, we use chitosan as our scaffold and employ its pH-responsive network-forming properties for vesicle restraint and its susceptibility to enzymatic cleavage for vesicle mobilization. These capabilities illustrate the versatility of chitosan and augment a growing list of attributes that chitosan offers for applications in foods,³² textiles,³³ catalysis,^{34,35} medicine,^{36,37} and biofabrication.^{38,39}

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Vesicles are technologically interesting structures because they allow molecules to be compartmentalized so that they can be transported through a fluid without mixing or dilution. In addition, the release of these molecules from the vesicles can be achieved with spatiotemporal control through a variety of convenient cues (e.g., pH, temperature, and light).⁴⁰ Thus, vesicles are under intense study for the targeted delivery of therapeutics⁴⁰ and the compartmentalization of reagents for microfluidic applications.^{41,42} Potentially, the work reported here provides an additional level of control for the use of vesicles; it allows these

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nano- and microcompartments to be locally restrained and mobilized in response to controllable cues. We envision that vesicles from a restrained “reservoir” could be controllably mobilized for circulation, either for medical or microfluidic applications. For medical applications, it is important to note that the pH cue responsible for vesicle restraint is physiologically convenient. Potentially, an acidic nonrestraining vesicle network would be injectable, whereas the physiological pH would provide the cue to induce a stronger gel network to restrain the vesicles. Furthermore, slow metabolism of the chitosan scaffold within the body could provide the cue to mobilize vesicles slowly. Obviously, more work is required to test these possibilities.

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